```
=> fil wpix
FILE 'WPIX' ENTERED AT 11:21:42 ON 21 MAY 2004
COPYRIGHT (C) 2004 THOMSON DERWENT

FILE LAST UPDATED:          20 MAY 2004      <20040520/UP>
MOST RECENT DERWENT UPDATE:     200432        <200432/DW>
DERWENT WORLD PATENTS INDEX SUBSCRIBER FILE, COVERS 1963 TO DATE

>>> FOR A COPY OF THE DERWENT WORLD PATENTS INDEX STN USER GUIDE,
    PLEASE VISIT:
 http://www.stn-international.de/training_center/patents/stn_guide.pdf <<<

>>> FOR DETAILS OF THE PATENTS COVERED IN CURRENT UPDATES, SEE
    http://thomsonderwent.com/coverage/latestupdates/           <<<

>>> FOR INFORMATION ON ALL DERWENT WORLD PATENTS INDEX USER
    GUIDES, PLEASE VISIT:
    http://thomsonderwent.com/support/userguides/               <<<

>>> NEW! FAST-ALERTING ACCESS TO NEWLY-PUBLISHED PATENT
    DOCUMENTATION NOW AVAILABLE IN DERWENT WORLD PATENTS INDEX
    FIRST VIEW - FILE WPIFV. FREE CONNECT HOUR UNTIL 1 MAY 2004.
    FOR FURTHER DETAILS: http://www.thomsonderwent.com/dwpifv  <<<

>>> NEW! IMPROVE YOUR LITIGATION CHECKING AND INFRINGEMENT
    MONITORING WITH LITALERT. FIRST ACCESS TO RECORDS OF IP
    LAWSUITS FILED IN THE 94 US DISTRICT COURTS SINCE 1973.
    FOR FURTHER DETAILS:
    http://www.thomsonscientific.com/litalert           <<<

>>> THE DISPLAY LAYOUT HAS BEEN CHANGED TO ACCOMODATE THE
    NEW FORMAT GERMAN PATENT APPLICATION AND PUBLICATION
    NUMBERS. SEE ALSO:
    http://www.stn-international.de/archive/stnews/news0104.pdf <<<

>>> SINCE THE FILE HAD NOT BEEN UPDATED BETWEEN APRIL 12-16
    THERE WAS NO WEEKLY SDI RUN  <<<

=> d all abeq tech abex tot l85
```

A waiting for translation

```
L85  ANSWER 1 OF 2  WPIX COPYRIGHT 2004 THOMSON DERWENT on STN
AN   2003-432116 [41]    WPIX
DNN  N2003-344917        DNC C2003-114390
TI   Vaccine formulation against bacterial, viral, mycotic, prion or
     parasitic infections, includes a combination of at least two paraben
     esters and 2-phenoxyethanol as preservative.
DC   B05 C03 P14
PA   (DAVE-N) DANMARKS VETERINAEINST
CYC  1
PI   DE 20219829     U1 20030508 (200341)*      13    A61K039-00     <--
ADT  DE 20219829 U1 DE 2002-20219829 20021220
PRAI DE 2002-20219829     20021220
IC   ICM  A61K039-00
     ICS  A01K047-06
AB   DE  20219829 U UPAB: 20030630
     NOVELTY - Vaccine formulation comprising an immunogen, a
     preservative and a carrier includes a combination of at least two paraben
     esters and 2-phenoxyethanol as the preservative.
          DETAILED DESCRIPTION - An INDEPENDENT CLAIM is also included for a
     stock solution for vaccine production comprising a preservative
     as above together with an aluminum hydroxide gel
     and/or quillaja saponin.
          ACTIVITY - Antibacterial; Virucide; Fungicide; Antiparasitic.
```

MECHANISM OF ACTION - **Vaccine.**

USE - The formulation is useful for **vaccinating** humans or other animals against bacterial, viral, mycotic, prion or parasitic infections (a veterinary **vaccine** comprising inactivated porcine parvovirus is specifically disclosed).

ADVANTAGE - The preservative is effective in preventing microbiological spoilage without impairing the immunogenic activity of the **vaccine.**

Dwg.0/0

FS CPI GMPI

FA AB; DCN

MC CPI: B04-A07E; B05-A01B; B05-A02; B07-A02; B12-M06; **B14-S11A**; **B14-S11B**; C04-A07E; C05-A01B; C05-A02; C12-M06; **C14-S11**

TECH UPTX: 20030630

TECHNOLOGY FOCUS - PHARMACEUTICALS - Preferred **Vaccine**: The formulation contains (mg/ml) **methyl paraben** (1.3), **propyl paraben** (0.2) and 2-**phenoxyethanol** (1). The carrier comprises diluents, stabilizers, adjuvants (especially **aluminum hydroxide** gel and/or Quil-A saponin), preservatives, buffers, surfactants, viscosity regulators and/or osmotic pressure regulators.

ABEX UPTX: 20030630

EXAMPLE - A preservative stock solution comprised **propyl paraben** (40 g), **methyl paraben** (60 g), 2-**phenoxyethanol** (200 g) and 96% ethanol (to 2000 ml). A veterinary **vaccine** comprised inactivated porcine parvovirus (21898 ml, 3 microg virions/ml), a 1.3% **aluminum hydroxide** hydrogel (67760 ml), 2 M glycine (319 ml), ultrafiltered water (32258 ml), phosphate-buffered saline (13540 ml, pH 7.2), 2% Quil A solution (1752 ml), 2 M sodium thiosulfate (680 ml), preservative stock solution (1400 ml) and antifoam (392 ml).

L85 ANSWER 2 OF 2 WPIX COPYRIGHT 2004 THOMSON DERWENT on STN 103 ant ✓

AN 1991-057987 [08] WPIX

DNC C1991-024461

TI New stable **vaccine** compsn. - comprises mixture of antigen and adjuvant amount of interleukin adsorbed onto mineral in suspension and preservative.

DC B04 D16

IN BIXLER, G; PILLAI, S

PA (PRAX-N) PRAXIS BIOLOGICS INC; (AMCY) AMERICAN CYANAMID CO; (PRAX-N) PRAXIS BIOLOGICS

CYC 20

PI	WO 9101143	A	19910207 (199108)*		32	
	RW: AT BE CH DE DK ES FR GB IT LU NL SE					
	W: AU CA FI JP KR NO					
	AU 9060500	A	19910222 (199120)			
	FI 9200132	A	19920113 (199215)			
	EP 482076	A	19920429 (199218)	EN	32	
	R: AT BE CH DE DK ES FR GB IT LI LU					
	NO 9200161	A	19920305 (199223)			A61K039-39 <--
	JP 04506663	W	19921119 (199301)		11	A61K039-39 <--
	AU 648509	B	19940428 (199422)			A61K039-39 <--
	EP 482076	B1	19950426 (199521)	EN	13	A61K039-39 <--
	R: AT BE CH DE DK ES FR GB IT LI LU NL SE					
	DE 69018990	E	19950601 (199527)			A61K039-39 <--
	ES 2075900	T3	19951016 (199547)			A61K039-39 <--
	NO 301577	B1	19971117 (199802)			A61K039-39 <--
	BR 1100816	A3	19980512 (199828)			A61K039-02 <--
	FI 104233	B1	19991215 (200005)			A61K039-39 <--
	KR 177179	B1	19990320 (200043)			A61K039-39 <--
	JP 2004002463	A	20040108 (200405)		15	A61K039-00 <--

JP 3485184 B2 20040113 (200410) 14 A61K039-39 <--
ADT EP 482076 A EP 1990-911344 19900716; NO 9200161 A WO 1990-US3982 19900716, NO 1992-161 19920113; JP 04506663 W JP 1990-510600 19900716, WO 1990-US3982 19900716; AU 648509 B AU 1990-60500 19900716; EP 482076 B1 EP 1990-911344 19900716, WO 1990-US3982 19900716; DE 69018990 E DE 1990-618990 19900716, EP 1990-911344 19900716, WO 1990-US3982 19900716; ES 2075900 T3 EP 1990-911344 19900716; NO 301577 B1 WO 1990-US3982 19900716, NO 1992-161 19920113; BR 1100816 A3 BR 1997-1100816 19970512; FI 104233 B1 WO 1990-US3982 19900716, FI 1992-132 19920113; KR 177179 B1 WO 1990-US3982 19900716, KR 1992-700086 19920113; JP 2004002463 A Div ex JP 1990-510600 19900716, JP 2003-284148 20030731; JP 3485184 B2 JP 1990-510600 19900716, WO 1990-US3982 19900716
FDT EP 482076 A Based on WO 9101143; JP 04506663 W Based on WO 9101143; AU 648509 B Previous Publ. AU 9060500, Based on WO 9101143; EP 482076 B1 Based on WO 9101143; DE 69018990 E Based on EP 482076, Based on WO 9101143; ES 2075900 T3 Based on EP 482076; NO 301577 B1 Previous Publ. NO 9200161; FI 104233 B1 Previous Publ. FI 9200132; JP 3485184 B2 Previous Publ. JP 04506663, Based on WO 9101143
PRAI US 1989-379742 19890714
REP 4.Jnl.Ref; EP 343480; EP 351876; 2.Jnl.Ref; GB 2217600
IC ICM **A61K039-00**; **A61K039-02**; **A61K039-39**
ICS A61K037-02; A61K038-00; A61K038-20; **A61K039-05**; **A61K039-07**; **A61K039-08**; **A61K039-085**; **A61K039-09**; **A61K039-095**; **A61K039-10**; **A61K039-102**; **A61K039-104**; **A61K039-106**; **A61K039-108**; **A61K039-12**; **A61K039-15**; **A61K039-21**; **A61K039-245**; **A61K039-385**; A61P031-04; A61P031-12; A61P035-00
AB WO 9101143 A UPAB: 20040205
The interleukin is preferably at least 1 of interleukin-1 alpha-1beta -2, -2, -4, -5, -6 and -7 and is especially human interleukin-2. The mineral suspension is an aqueous suspension of **alum**. The anitgen is selected from bacteria, viruses, macro-components of cells, proteins, peptides, glycoproteins, carbohydrates, parasites, fungi, oncogene products and cancer cells. The bacterial antigen is from a bacterial pathogen e.g. Haemophilus influenzae. The antigen is coupled to a glycoconjugate comprising a bacterial toxin of diphtheria, tetenus, pertussis or CRN etc. The preservative is thimerosal, phenol, benzyl alcohol, **ethyl**- or **ethyl paraben** 2 **phenoxyethanol** or m-cresol.
USE/ADVANTAGE: For producing an immune response to an antigen in a vertebrate, for preventing microbial infections and for treating AIDS.
Dwg.0/0
FS CPI
FA AB; DCN
MC CPI: **B02-V02**; B04-D02; B12-M06; D05-H07; D05-H09
ABEQ EP 482076 B UPAB: 19950602
A stable **vaccine** composition, comprising a mixt. of an antigen and an adjuvant-1beta, interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, interleukin-7, or mixts. thereof, absorbed onto an aqueous suspension of **alum** (e.g. **aluminium** hydroxide, or **aluminium** phosphate) and a pharmaceutically acceptable preservative, in a pharmaceutically accpetable vehicle and optional adjuvant.
Dwg.0/0

=> d all abeq tech abex l86

L86 ANSWER 1 OF 1 WPIX COPYRIGHT 2004 THOMSON DERWENT on STN
AN 2000-160229 [14] WPIX
DNC C2000-049970
TI Cosmetic skin care composition, useful e.g. for reducing oily skin and

sebum secretion, contains a low molecular weight fraction from gugulipid.
DC B04 D21
IN BAJOR, J S; TALLMAN, M
PA (ARDE-N) ARDEN CO DIV CONOPCO INC ELIZABETH
CYC 1
PI US 6019975 A 20000201 (200014)* 9 A61K035-78
ADT US 6019975 A US 1997-969263 19971113
PRAI US 1997-969263 19971113
IC ICM A61K035-78
ICS **A61K039-385**
AB US 6019975 A UPAB: 20000320
NOVELTY - Cosmetic skin care composition comprises:
(a) 0.0001-5 % of a low molecular weight fraction of gugulipid of molecular weight less than 500 Daltons (Da) as an anti-sebum agent; and (b) a vehicle.
The low molecular weight fraction is obtained by dispersing or dissolving gugulipid in a polar solvent, separating by ultrafiltration and concentrating the filtrate.
ACTIVITY - Dermatological; anti-sebum.
A 6.5% solution of gugulipid extract in methanol was filtered through a 500 Da filter and evaporated to give an extract. Cultured human sebaceous glands were incubated with the extract at 37 deg. C for 30 minutes and sebum production was assayed. The low molecular weight fraction at 0.01% reduced sebum secretion by 47.9% and at 0.04% by 54.5%. Gugulipid at 0.01% reduced sebum secretion by 50.9%.
MECHANISM OF ACTION - Antioxidant.
USE - The composition is useful for reducing or preventing oily skin, reducing sebum secretion and protecting the skin from free radical activity (claimed). The composition prevents shine and stickiness, reduces the appearance of wrinkles and aged skin, improves skin colour, radiance, clarity and finish, and gives an overall youthful appearance. It is particularly useful for application to the face, but may also be used on the neck, chest, back and scalp.
Dwg.0/0
FS CPI
FA AB; DCN
MC CPI: B04-A10; B04-B01B; B14-N17; B14-R01; B14-S08; D08-B09A
ABEX UPTX: 20000320
EXAMPLE - An oil controlling lotion was prepared comprising (% by weight): propylene glycol (1.0); xanthan gum (0.20); disodium N,N'-1,2-ethanediylbisN-carboxymethyl)glycine (EDTA; 0.10); **methyl paraben** (0.30); polysorbate 20 (1.50); **octyl** methoxycinnamate (2.0); low molecular weight fraction of gugulipid (0.001); cetyl alcohol (1.50); polyethylene glycol 165 glycerol stearate (3.0); **propyl paraben** (0.10); cyclomethicone (15.0); dimethicone (2.0); dimethiconol (0.50); micronized titanium dioxide (0.50); sodium hyaluronate (1% solution; 3.0); triethanolamine (99%; 0.20); salicylic acid (0.20); **phenoxyethanol** (0.35); and water (q.s.).

Table

=> fil hcaplus
FILE 'HCAPLUS' ENTERED AT 11:21:58 ON 21 MAY 2004
USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.
PLEASE SEE "HELP USAGETERMS" FOR DETAILS.

FILE COVERS 1907 - 21 May 2004 VOL 140 ISS 22
FILE LAST UPDATED: 20 May 2004 (20040520/ED)

This file contains CAS Registry Numbers for easy and accurate substance identification.

=> d all hitstr tot 166

L66 ANSWER 1 OF 4 HCAPLUS COPYRIGHT 2004 ACS on STN
AN 2003:451780 HCAPLUS
DN 139:12330
ED Entered STN: 13 Jun 2003
TI **Vaccine** formulations containing at least two paraben esters and **phenoxyethanol** as preservatives
PA Danmarks Veterinaeinstitut, Den.
SO Ger. Gebrauchsmusterschrift, 13 pp.
CODEN: GGXXFR
DT Patent
LA German
IC ICM A61K039-00
ICS A01K047-06
CC 63-6 (Pharmaceuticals)
Section cross-reference(s): 15
FAN.CNT 1

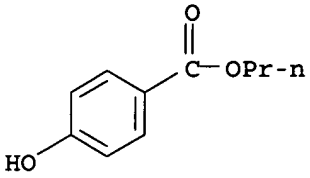
PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI DE 20219829	U1	20030612	DE 2002-20219829	20021220
PRAI DE 2002-20219829		20021220		

AB The invention concerns **vaccines** that contain immunogens and adjuvants in pharmaceutical acceptable carriers with preservative mixts. composed of at least two paraben esters and **phenoxyethanol**. Thus 2 L of ethanolic (96% ethanol) preservative solution were prepared containing (g): **propyl-4-hydroxybenzoate** 40; **Methyl-4-hydroxybenzoate** 60; 2-**phenoxyethanol** 200. The solution was used as a 1400 mL component in a veterinary **vaccine** that further included (mL): inactivated porcine parvovirus (3 μg Virion/mL) 21898; **alhydrogel** (1.3% **Al2(OH)3**) 67760; 2 M glycine 319, water 32258; phosphate-NaCl, pH 7.2 13540; 2% quil A solution 1752; 2 M sodium thiosulfate 680; antifoaming agent 392.
ST **vaccine** injection preservative **phenoxyethanol** paraben ester
IT Immunostimulants
(adjuvants; **vaccine** formulations containing at least two paraben esters and **phenoxyethanol** as preservatives)
IT Drug delivery systems
(injections; **vaccine** formulations containing at least two paraben esters and **phenoxyethanol** as preservatives)
IT Preservatives
Vaccines
(**vaccine** formulations containing at least two paraben esters and **phenoxyethanol** as preservatives)
IT Porcine parvovirus
(**vaccine**; **vaccine** formulations containing at least two paraben esters and **phenoxyethanol** as preservatives)
IT **94-13-3**, **Propyl-4-hydroxybenzoate**

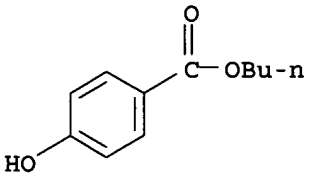
94-26-8, Butyl paraben 99-76-3, Methyl-4-hydroxybenzoate 99-96-7D, alkyl esters, esters **120-47-8, Ethyl paraben 122-99-6, 2-Phenoxyethanol 21645-51-2, Aluminum hydroxide (Al(OH)3)**, biological studies 66594-14-7, Quil-A
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(**vaccine** formulations containing at least two **paraben** esters and **phenoxyethanol** as preservatives)

IT **94-13-3, Propyl-4-hydroxybenzoate 94-26-8, Butyl paraben 99-76-3, Methyl-4-hydroxybenzoate 120-47-8, Ethyl paraben 122-99-6, 2-Phenoxyethanol 21645-51-2, Aluminum hydroxide (Al(OH)3)**, biological studies
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(**vaccine** formulations containing at least two **paraben** esters and **phenoxyethanol** as preservatives)

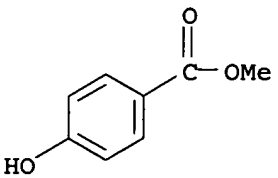
RN 94-13-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, propyl ester (9CI) (CA INDEX NAME)



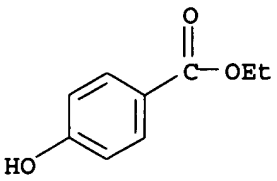
RN 94-26-8 HCAPLUS
CN Benzoic acid, 4-hydroxy-, butyl ester (9CI) (CA INDEX NAME)



RN 99-76-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, methyl ester (9CI) (CA INDEX NAME)



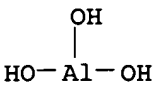
RN 120-47-8 HCAPLUS
CN Benzoic acid, 4-hydroxy-, ethyl ester (9CI) (CA INDEX NAME)



RN 122-99-6 HCAPLUS
CN Ethanol, 2-phenoxy- (6CI, 7CI, 8CI, 9CI) (CA INDEX NAME)

PhO—CH_2—CH_2—OH

RN 21645-51-2 HCAPLUS
CN Aluminum hydroxide (Al(OH)3) (9CI) (CA INDEX NAME)

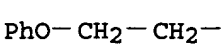


L66 ANSWER 2 OF 4 HCAPLUS COPYRIGHT 2004 ACS on STN — Done
AN 1998:548516 HCAPLUS
DN 129:180138
ED Entered STN: 28 Aug 1998
TI Thimerosal-free preservatives for **vaccines**
IN Ng, Assunta S.; Hennessey, John P.; Mancinelli, Ralph J.
PA Merck & Co., Inc., USA
SO PCT Int. Appl., 25 pp.
CODEN: PIXXD2
DT Patent
LA English
IC ICM A61K009-08
ICS A61K039-02; A61K039-12; A61K047-10; A61K047-14
CC 63-6 (Pharmaceuticals)
FAN.CNT 1

	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9834594	A1	19980813	WO 1998-US2283	19980203
	W: CA, JP, US				
	RW: AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE				
	EP 971696	A1	20000119	EP 1998-906181	19980203
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, PT, IE, FI				
	JP 2002515056	T2	20020521	JP 1998-534902	19980203
PRAI	US 1997-36900P	P	19970206		
	WO 1998-US2283	W	19980203		

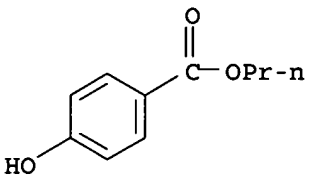
AB Novel combination of preservatives (**Me** and **Pr** **parabens**, benzyl alc., and 2-**phenoxyethanol**) were found to pass antimicrobial testing according to USP, BP, and EP. The new preservatives were put into **vaccines** using L-histidine as a buffer to keep pH at 7.0. HPLC methods were developed to analyze these preservatives and their degradation products.
ST preservative **vaccine**; paraben preservative **vaccine**
IT HPLC
Preservatives
Vaccines
(thimerosal-free preservatives for **vaccines**)
IT 71-00-1, L-Histidine, biological studies

RL: MOA (Modifier or additive use); THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(buffer; thimerosal-free preservatives for **vaccines**)
IT 100-51-6, Benzyl alcohol, biological studies **122-99-6**, 2-**Phenoxyethanol** 5026-62-0, Benzoic acid, 4-hydroxy-, methyl ester, sodium salt 35285-69-9, Benzoic acid, 4-hydroxy-, propyl ester, sodium salt
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(thimerosal-free preservatives for **vaccines**)
RE.CNT 7 THERE ARE 7 CITED REFERENCES AVAILABLE FOR THIS RECORD
RE
(1) American Cyanamid Company; EP 0750907 A2 1997 HCAPLUS
(2) Cameron, J; Develop Biol Standard 1974, V24, P155 HCAPLUS
(3) Dwyer; US 5603933 A 1997 HCAPLUS
(4) Kneczke, M; Determination of Pilocarpine, Physostigmine, its Degredation Product Reserine and Preservatives by High Performance Liquid Chromatography 1980, V198, P529 HCAPLUS
(5) Lowe, I; Let Appl Microbiol 1994, V18, P115 HCAPLUS
(6) Monath, T; Develop Biol Standard 1996, V87, P219 MEDLINE
(7) Parker; US 5672350 A 1997 HCAPLUS
IT **122-99-6**, 2-**Phenoxyethanol**
RL: THU (Therapeutic use); BIOL (Biological study); USES (Uses)
(thimerosal-free preservatives for **vaccines**)
RN 122-99-6 HCAPLUS
CN Ethanol, 2-phenoxy- (6CI, 7CI, 8CI, 9CI) (CA INDEX NAME)

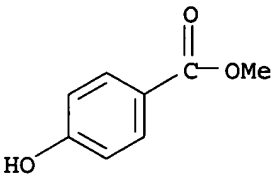


L66 ANSWER 3 OF 4 HCAPLUS COPYRIGHT 2004 ACS on STN
AN 1975:103063 HCAPLUS
DN 82:103063
ED Entered STN: 12 May 1984
TI Preservative systems compatible with DPT (diphtheria, pertussis, tetanus)-polio (Salk) and TABTD [typhoid A B, tetanus, diphtheria]-polio (Salk) **vaccines**
AU Cameron, Jack
CS Connaught Lab. Ltd., Toronto-Willowdale, ON, Can.
SO Developments in Biological Standardization (1974), 24, 155-65
CODEN: DVBSA3; ISSN: 0301-5149
DT Journal
LA English
CC 63-3 (Pharmaceuticals)
AB A review is given of different preservative systems compatible with the title products with particular reference to the 2-**phenoxyethanol** [**122-99-6**], neomycin [1404-04-2], streptomycin [57-92-1] combination which has proved to be satisfactory. Extended data on the stability of DPT-polio and TABTD-polio preserved with this system are also given.
ST preservative polio **vaccine** compatibility
IT Bactericides, Disinfectants and Antiseptics
Fungicides and Fungistats
Preservatives
(for polio **vaccines**, compatibility of)
IT Bordetella pertussis
Clostridium tetani
Corynebacterium diphtheriae
Salmonella typhi
(polio **vaccine** containing, preservatives for, compatibility of)
IT **Vaccines**
(polio, DPT- and TABTD-, preservatives for, compatibility of)

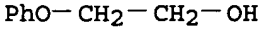
IT Virus, animal
(polio-, **vaccines** of, preservatives for, compatibility of)
IT 57-92-1, biological studies
RL: BIOL (Biological study)
(preservative with neomycin and **phenoxyethanol**, for polio **vaccine**)
IT 1404-04-2
RL: BIOL (Biological study)
(preservative with **phenoxyethanol** and streptomycin, for polio **vaccine**)
IT 50-00-0, biological studies **94-13-3 99-76-3** 121-54-0 **122-99-6**
RL: BIOL (Biological study)
(preservative, for **vaccines**)
IT **94-13-3 99-76-3 122-99-6**
RL: BIOL (Biological study)
(preservative, for **vaccines**)
RN 94-13-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, propyl ester (9CI) (CA INDEX NAME)



RN 99-76-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, methyl ester (9CI) (CA INDEX NAME)



RN 122-99-6 HCAPLUS
CN Ethanol, 2-phenoxy- (6CI, 7CI, 8CI, 9CI) (CA INDEX NAME)



L66 ANSWER 4 OF 4 HCAPLUS COPYRIGHT 2004 ACS on STN
AN 1975:103061 HCAPLUS
DN 82:103061
ED Entered STN: 12 May 1984
TI Compatibility of various preservatives with live virus **vaccines**
AU Gray, Alan; Schuchardt, L. F.; Hanson, H. J.
CS Merck Sharp and Dohme, West Point, PA, USA
SO Developments in Biological Standardization (1974), 24, 123-9
CODEN: DVBSA3; ISSN: 0301-5149
DT Journal
LA English
CC 63-3 (Pharmaceuticals)
AB A large number of commonly used preservatives were screened for compatibility with measles and rubella viruses. Thimerosal [54-64-8] and Quatresin

[2748-88-1] with and without EDTA [60-00-4] for sterilization were studied. The effective concns. for a number of preservatives against representative test organisms were also determined

ST preservative compatibility virus **vaccine**

IT Quaternary ammonium compounds, biological studies
RL: BIOL (Biological study)
(alkyldimethyl(phenylmethyl), chlorides, preservatives for virus **vaccines**, compatibility of)

IT Preservatives
(for virus **vaccines**, compatibility of)

IT Virus, animal
(measles and rubella, **vaccines** of, preservatives for, compatibility of)

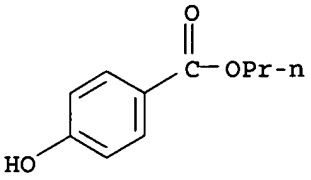
IT **Vaccines**
(preservatives for, compatibility of)

IT Measles
Rubella
(virus **vaccines** of, preservatives for, compatibility of)

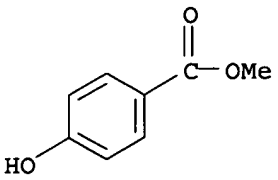
IT 54-64-8 55-56-1 57-09-0 57-15-8 59-50-7 60-00-4, biological studies 60-12-8 70-30-4 **94-13-3 99-76-3** 100-51-6 102-98-7 121-54-0 122-18-9 **122-99-6** 123-03-5 136-77-6 2748-88-1 25155-18-4
RL: BIOL (Biological study)
(preservative, for virus **vaccines**, compatibility of)

IT **94-13-3 99-76-3 122-99-6**
RL: BIOL (Biological study)
(preservative, for virus **vaccines**, compatibility of)

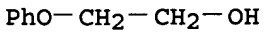
RN 94-13-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, propyl ester (9CI) (CA INDEX NAME)



RN 99-76-3 HCAPLUS
CN Benzoic acid, 4-hydroxy-, methyl ester (9CI) (CA INDEX NAME)



RN 122-99-6 HCAPLUS
CN Ethanol, 2-phenoxy- (6CI, 7CI, 8CI, 9CI) (CA INDEX NAME)



=> fil reg
FILE 'REGISTRY' ENTERED AT 11:22:15 ON 21 MAY 2004
USE IS SUBJECT TO THE TERMS OF YOUR STN CUSTOMER AGREEMENT.

PLEASE SEE "HELP USAGETERMS" FOR DETAILS.

Property values tagged with IC are from the ZIC/VINITI data file provided by InfoChem.

STRUCTURE FILE UPDATES: 20 MAY 2004 HIGHEST RN 684211-73-2
DICTIONARY FILE UPDATES: 20 MAY 2004 HIGHEST RN 684211-73-2

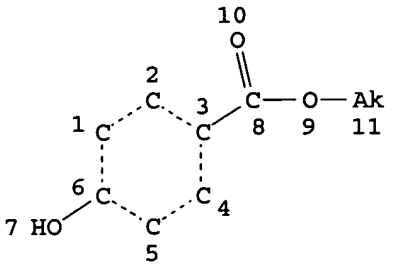
TSCA INFORMATION NOW CURRENT THROUGH JANUARY 6, 2004

Please note that search-term pricing does apply when conducting SmartSELECT searches.

Crossover limits have been increased. See HELP CROSSOVER for details.

Experimental and calculated property data are now available. For more information enter HELP PROP at an arrow prompt in the file or refer to the file summary sheet on the web at:
http://www.cas.org/ONLINE/DBSS/registryss.html

=> d sta que l21
L9 4 SEA FILE=REGISTRY ABB=ON PLU=ON 99-76-3 OR 120-47-8 OR 94-13-3 OR 94-26-8
L10 3 SEA FILE=REGISTRY ABB=ON PLU=ON 6521-29-5 OR 1083-27-8 OR 1085-12-7
L11 STR



NODE ATTRIBUTES:
DEFAULT MLEVEL IS ATOM
DEFAULT ECLEVEL IS LIMITED

GRAPH ATTRIBUTES:
RSPEC 1
NUMBER OF NODES IS 11

STEREO ATTRIBUTES: NONE
L13 567 SEA FILE=REGISTRY CSS FUL L11
L14 139 SEA FILE=REGISTRY ABB=ON PLU=ON L13 AND 1/NC
L15 10 SEA FILE=REGISTRY ABB=ON PLU=ON L14 AND IDS/CI
L16 2 SEA FILE=REGISTRY ABB=ON PLU=ON L15 AND (C23H38O3 OR C20H32O3 OR C17H16O3)
L17 129 SEA FILE=REGISTRY ABB=ON PLU=ON L14 NOT L15
L18 131 SEA FILE=REGISTRY ABB=ON PLU=ON (L17 OR L16)
L19 113 SEA FILE=REGISTRY ABB=ON PLU=ON L18 NOT (11C# OR 13C# OR 14C# OR C11# OR C13# OR C14# OR (D OR T)/ELS OR LABELED OR ION OR CONJUGATE OR 18O# OR 17O#)
L20 108 SEA FILE=REGISTRY ABB=ON PLU=ON L19 NOT PMS/CI
L21 108 SEA FILE=REGISTRY ABB=ON PLU=ON (L9 OR L10 OR L20)

=> d his

```
     (FILE 'REGISTRY' ENTERED AT 10:35:07 ON 21 MAY 2004)
               DEL HIS

     FILE 'HCAPLUS' ENTERED AT 10:35:28 ON 21 MAY 2004
L1           831 S METHYL PARABEN
L2           193 S ETHYL PARABEN
L3           570 S PROPYL PARABEN
L4           145 S BUTYL PARABEN
L5            10 S (PENTYL OR HEXYL OR HEPTYL OR OCTYL)()PARABEN
L6          1303 S (METHYL OR ETHYL OR PROPYL OR BUTYL OR PENTYL OR HEXYL OR BUT
L7          4512 S METHYLPARABEN OR ETHYLPARABEN OR PROPYLPARABEN OR BUTYLPARABE
L8            17 S PENTYLPARABEN OR HEXYLPARABEN OR HEPTYLPARABEN OR OCTYLPARABE

     FILE 'REGISTRY' ENTERED AT 10:41:40 ON 21 MAY 2004
L9             4 S 99-76-3 OR 120-47-8 OR 94-13-3 OR 94-26-8
L10            3 S 6521-29-5 OR 1083-27-8 OR 1085-12-7
L11              STR
L12            3 S L11 CSS SAM
L13          567 S L11 CSS FUL
                 SAV L13 LEPARABEN/A
L14          139 S L13 AND 1/NC
L15           10 S L14 AND IDS/CI
L16            2 S L15 AND (C23H38O3 OR C20H32O3 OR C17H16O3)
L17          129 S L14 NOT L15
L18          131 S L17,L16
L19          113 S L18 NOT (11C# OR 13C# OR 14C# OR C11# OR C13# OR C14# OR (D O
L20          108 S L19 NOT PMS/CI
L21          108 S L9,L10,L20
                 E ALUMINUM HYDROXIDE/CN
L22            1 S E3
L23            2 S E3,E9
L24            2 S L22,L23
L25            1 S ALH4O4/MF NOT CCS/CI
L26            3 S L24,L25
                 E AL2O3/MF
L27            8 S E3
L28            1 S L27 NOT (TIS OR MNS OR CCS)/CI

     FILE 'HCAPLUS' ENTERED AT 10:51:22 ON 21 MAY 2004
L29         7448 S L21
L30         4749 S (METHYL OR ETHYL OR PROPYL OR BUTYL)()(P OR 4)()(HYDROXYBENZO
L31            9 S (P OR 4)()(HYDROXYBENZOIC OR HYDROXY BENZOIC)()ACID()(METHYLE
L32          303 S (P OR 4)()(HYDROXYBENZOIC OR HYDROXY BENZOIC)()ACID()(METHYL
L33            0 S (P OR 4)()(HYDROXYBENZOATE OR HYDROXY BENZOATE)()(METHYLESTER
L34            2 S (P OR 4)()(HYDROXYBENZOATE OR HYDROXY BENZOATE)()(METHYL OR E
L35        13381 S L1-L8,L29-L34
L36        21821 S L26
L37       224019 S L28
L38        22873 S (AL OR ALUMINUM)()HYDROXIDE
L39         1548 S (ALUMINUM OR ALUMINA)()(TRIHYDRATE OR TRIHYDROXIDE)
L40        21348 S AL OH 3
L41          538 S AL OH 2
L42         1846 S HIGILITE OR HYDRATED ALUMINA
L43           83 S L35 AND L36
L44          124 S L35 AND L38-L42
L45          155 S L35 AND (L37 OR AL2O3 OR ALUMINA OR (AL OR ALUMINUM)()(TRIOXI
L46          269 S L43-L45
L47            2 S L35 AND (ALHYDROGEL OR AL2 OH 3)
L48          269 S L46,L47

     FILE 'REGISTRY' ENTERED AT 11:01:20 ON 21 MAY 2004
L49            1 S 2-PHENOXYETHANOL/CN
```

```
     FILE 'HCAPLUS' ENTERED AT 11:02:29 ON 21 MAY 2004
L50         2456 S L49
L51         2217 S PHENOXYETHANOL OR PHENOXYETHYL ALCOHOL
L52          512 S L35 AND L50,L51
L53           13 S L52 AND L48
L54            1 S L53 AND VACCINE
L55            1 S L46 AND VACCINE
L56           34 S L35 AND VACCINE
L57            4 S L56 AND L50,L51
L58            4 S L54,L55,L57
                 E VACCINE/CT
L59        38049 S E4-E29
                 E E4+ALL
                 E VACCINATION/CT
                 E E3+ALL
L60         2852 S E1,E2
                 E IMMUNIZATION/CT
L61         7207 S E3-E7
                 E E3+ALL
L62         7370 S E3+NT
L63           33 S L35 AND L59-L62
L64            4 S L63 AND L52
L65            1 S L63 AND L48
L66            4 S L58,L64,L65

     FILE 'WPIX' ENTERED AT 11:07:39 ON 21 MAY 2004
                 E DE2002-20219829/AP,PRN
L67            1 S E3
L68         3154 S L1/BIX OR L2/BIX OR L3/BIX OR L4/BIX OR L5/BIX OR L6/BIX OR L
                 E METHYL PARABEN/DCN
                 E METHYLPARABEN/DCN
                 E E3+ALL
L69         1339 S E2 OR 0689/DRN
                 E ETHYLPARABEN/DCN
                 E E3+ALL
L70          197 S E2
                 E PROPYLPARABEN/DCN
                 E E3+ALL
L71          846 S E2 OR 0607/DRN
                 E BUTYLPARABEN/DCN
                 E BUTYL PARABEN/DCN
                 E R17233+ALL/DCN
L72           44 S E1
                 E R10246+ALL/DCN
                 E R02020+ALL/DCN
L73         3912 S L68-L72
L74        14789 S E1 OR 2020/DRN OR L38/BIX OR L39/BIX OR L40/BIX OR L41/BIX OR
L75           85 S L73 AND L74
L76          917 S L51/BIX
L77           99 S R10245/DCN
L78           14 S L75 AND L76,L77
L79          307 S L73 AND L76,L77
L80            2 S L78,L79 AND VACCIN?/BIX
L81            3 S L79 AND A61K039/IC,ICM,ICS
L82            2 S L79 AND (B14-S11? OR C14-S11? OR B02-V02 OR C02-V02)/MC
L83            3 S L67,L80-L82
L84            3 S L83 AND L67-L83
L85            2 S L84 AND ALUM?/BIX
L86            1 S L84 NOT L85

     FILE 'WPIX' ENTERED AT 11:21:42 ON 21 MAY 2004
```

FILE 'HCAPLUS' ENTERED AT 11:21:58 ON 21 MAY 2004

FILE 'REGISTRY' ENTERED AT 11:22:15 ON 21 MAY 2004

=>

JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **24**, 5–13 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jat.958

Concentrations of Parabens in Human Breast Tumours

P. D. Darbre,[1,*] A. Aljarrah,[2] W. R. Miller,[2] N. G. Coldham,[3] M. J. Sauer[4] and G. S. Pope[1]

[1] Division of Cell and Molecular Biology, School of Animal and Microbial Sciences, University of Reading, Reading RG6 6AJ, UK
[2] The Edinburgh Breast Unit Research Group, Paderewski Building, Western General Hospital, Edinburgh EH4 2XU, UK
[3] Department of Bacterial Diseases, Veterinary Laboratories Agency, Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK
[4] Department of TSE Molecular Biology, Veterinary Laboratories Agency, Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK

Key words: parabens; xenoestrogens; oestrogenic activity; HPLC–MS–MS; human breast cancer; preservatives; cosmetics.

Parabens are used as preservatives in many thousands of cosmetic, food and pharmaceutical products to which the human population is exposed. Although recent reports of the oestrogenic properties of parabens have challenged current concepts of their toxicity in these consumer products, the question remains as to whether any of the parabens can accumulate intact in the body from the long-term, low-dose levels to which humans are exposed. Initial studies reported here show that parabens can be extracted from human breast tissue and detected by thin-layer chromatography. More detailed studies enabled identification and measurement of mean concentrations of individual parabens in samples of 20 human breast tumours by high-pressure liquid chromatography followed by tandem mass spectrometry. The mean concentration of parabens in these 20 human breast tumours was found to be 20.6 ± 4.2 ng g^{-1} tissue. Comparison of individual parabens showed that methylparaben was present at the highest level (with a mean value of 12.8 ± 2.2 ng g^{-1} tissue) and represents 62% of the total paraben recovered in the extractions. These studies demonstrate that parabens can be found intact in the human breast and this should open the way technically for more detailed information to be obtained on body burdens of parabens and in particular whether body burdens are different in cancer from those in normal tissues. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

The alkyl esters of *p*-hydroxybenzoic acid (parabens) are used widely as preservatives in many thousands of cosmetic, food and pharmaceutical products (Elder, 1984). These simple esters have proved to be very effective antimicrobial agents, with antimicrobial activity increasing with the length of the alkyl grouping from methyl to *n*-butyl (Murrell & Vincent, 1950), and it is the simplicity and effectiveness of these compounds that have resulted in their widespread use. As such, the human population is exposed to parabens from a wide variety of sources on a daily basis. Parabens are permitted as preservatives in food up to 0.1% and the average daily intake of parabens from food by adult humans was estimated in 1984 to be 4–6 mg kg^{-1} (Elder, 1984). In cosmetics, parabens are permitted in concentrations of up to 1% (Elder, 1984). In 1984, it was estimated that parabens were used in 13 200 different cosmetic formulations (Elder, 1984) and a more recent survey of 215 cosmetic products found that parabens were used in 99% of leave-on products and in 77% of rinse-off cosmetics (Rastogi *et al.*, 1995).

Animal studies have shown that parabens are rapidly absorbed, metabolized and excreted. Parabens are quickly absorbed from the gastrointestinal tract and from blood, hydrolysed to *p*-hydroxybenzoic acid, conjugated and the conjugate excreted in the urine (Jones *et al.*, 1956; Heim *et al.*, 1957; Tsukamoto & Terada, 1960, 1962, 1964; Derache & Gourdon, 1963; Phillips *et al.*, 1978; Kiwada *et al.*, 1979). Parabens also can be absorbed rapidly through intact skin (Whitworth & Jun, 1973; Fischmeister *et al.*, 1975; Komatsu & Suzuki, 1979) and this can be influenced by the presence of penetration enhancers found in cosmetic preparations (Kitagawa *et al.*, 1997). However, the presence of carboxylesterases in skin and subcutaneous fatty tissues results in varying hydrolysis to *p*-hydroxybenzoic acid (Lobemeier *et al.*, 1996) and this can also influence absorption (Bando *et al.*, 1997). However, the question remains as to whether any of the parabens can enter the body intact from the long-term, low-dose levels to which humans are exposed. Parabens have a high oil/water partition coefficient and water solubility decreases with increase in ester chain length (Elder, 1984). Therefore, if any parabens do enter the human body intact, they may be able to accumulate in fatty components of body tissues in a similar manner to that of other lipophilic

* Correspondence to: Dr. P. D. Darbre, Division of Cell and Molecular Biology, School of Animal and Microbial Sciences, University of Reading, PO Box 228, Whiteknights, Reading RG6 6AJ, UK.
E-mail: p.d.darbre@reading.ac.uk
Contract/grant sponsor: Seedcorn Fund of the Veterinary Laboratories Agency.

Received 23 June 2003
Revised 7 October 2003
Accepted 8 October 2003

pollutants that are known to bioaccumulate (Dobson *et al.*, 1989; Dobson, 1993; Sonawane, 1995; Hardell *et al.*, 1996; Guttes *et al.*, 1998; Stellman *et al.*, 1998, 2000; Darbre, 1998).

Most studies have indicated that parabens are not mutagenic (Elder, 1984), but there are reports that they can cause chromosomal aberrations (Ishidate *et al.*, 1978), particularly in the co-presence of polychlorinated biphenyls (Matsuoka *et al.*, 1979), and subcutaneous administration of methylparaben has been reported to cause mammary adenocarcinomas in rats (Mason *et al.*, 1971). At a cellular level, parabens have been shown capable of disrupting cellular function through inhibiting secretion of lysosomal enzymes (Bairati *et al.*, 1994) and causing mitochondrial dysfunction (Nakagawa & Maldeus, 1998). However, the recent discovery that parabens possess oestrogenic activity has challenged the concepts of their toxicity in new ways. Because parabens can bind to oestrogen receptors, they may be able to mediate unwanted effects at much lower concentrations and more specifically than through non-receptor mediated mechanisms.

The oestrogenic activity of parabens was first reported in 1998 (Routledge *et al.*, 1998). Since then, parabens have been shown to bind to oestrogen receptors from different sources, including rodent uterus (Routledge *et al.*, 1998; Blair *et al.*, 2000; Fang *et al.*, 2001) and MCF7 human breast cancer cells (Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003). They have been shown to regulate oestrogen-responsive reporter gene expression in yeast cells (Routledge *et al.*, 1998; Jin-Sung *et al.*, 2000; Nishihara *et al.*, 2000) and in human breast cancer cells (Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003), and expression of the endogenous oestrogen-regulated genes pS2 (Byford *et al.*, 2002) and progesterone receptor (Okubo *et al.*, 2001) in breast cancer cells. Parabens can increase the growth of MCF7 human breast cancer cells (Okubo *et al.*, 2001; Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003), which can be blocked with the antioestrogen ICI 182 780 (faslodex) (Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003), demonstrating the growth effects to be oestrogen-receptor-mediated. Their oestrogenic activity has been demonstrated also in animal models *in vivo* in fish (Pedersen *et al.*, 2000) and in increasing uterine weight in immature rats (Routledge *et al.*, 1998) and immature mice (Darbre *et al.*, 2002, 2003). In line with other environmental oestrogens, butylparaben has been shown also to be able to alter reproductive function in male rats, including reduction in sperm counts (Oishi, 2001). In general, the oestrogenic and antimicrobial activities of the parabens increase with the length and branching of the alkyl ester (Darbre *et al.*, 2002, 2003).

Because oestrogen is known to influence the incidence of breast cancer (Lipworth, 1995) and ablation of oestrogen action remains the preferred treatment for hormone-sensitive breast tumours (Miller, 1996), the presence of oestrogenic chemicals in the breast area could potentially influence both the incidence and treatment of breast cancer. Parabens are used as preservatives in a range of cosmetics applied to the underarm and breast area and it has been suggested that regular application of such oestrogenic chemicals could influence breast cancer development (Darbre, 2001, 2003; Harvey, 2003). However, the outstanding question remains as to whether parabens can enter and accumulate in the human breast. Previous studies have identified other environmental oestrogenic chemicals that can accumulate in fatty tissue of the breast (Dobson, 1993; Hardell *et al.*, 1996; Guttes *et al.*, 1998; Stellman *et al.*, 1998, 2000). This study has aimed to investigate whether parabens also can be detected in human breast tissue, using available breast tumour material. Initial experiments enabled the extraction of total parabens from human breast tissue to be visualized by thin-layer chromatography. More detailed studies enabled identification and measurement of individual parabens in human breast tumour samples by high-pressure liquid chromatography (HPLC) followed by tandem mass spectrometry (MS/MS).

MATERIALS AND METHODS

Human breast tumour material

Samples of human breast tumour material were collected at the Edinburgh Breast Unit and stored in liquid nitrogen.

Chemical standards

Methylparaben, ethylparaben, *n*-propylparaben, *n*-butylparaben and benzylparaben were purchased from Sigma (Poole, UK). Isobutylparaben was a gift from Nipa Laboratories (Mid-Glamorgan, UK). All compounds were made as stock solutions of 0.1 M in ethanol.

Extraction of parabens from human breast material and analysis by thin-layer chromatography

All glassware was pre-washed in 0.1 M NaOH and extractions were performed using sterile polycarbonate tubes (Falcon). Samples of human breast tissue (1 g) were chopped finely with a sterile razor and homogenized in 5 ml of hexane using a hand-homogenizer. Samples were left in a sealed polycarbonate tube with mixing for 1 h and then spun at 1500 rpm in a bench centrifuge at room temperature for 2 min. The supernatant was placed in a sterile polycarbonate tube, 5 ml of 0.1 M potassium bicarbonate was added and the tube was inverted 40 times by hand. The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The upper yellow hexane layer containing phenolic compounds was placed in a new sterile polycarbonate tube, 5 ml of 0.1 M potassium carbonate was added and again the tube was inverted 40 times by hand. The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The lower aqueous layer containing the phenols as potassium salts was taken into a new sterile polycarbonate tube and acidified by the addition of 300 µl of concentrated hydrochloric acid to give a pH in the 1–3 range (checked with pH paper). The free phenolic compounds released on acidification were extracted into 5 ml of diethyl ether by inverting the tube by hand 40 times (Pope *et al.*, 1990). The mixture was spun at 1500 rpm at room temperature for 2 min to separate the phases. The upper ether layer was removed and evaporated to dryness under nitrogen overnight in a fume hood.

The extract was taken up in 50 µl of ethanol and aliquots were run against paraben standards (50–400 ng per track) on thin-layer chromatography plates (DC-Alufolien Kieselgel 60 F254, Merck; ca. 6 cm wide × 8 cm high) using a solvent of 5% (v/v) ethanol–95% (v/v) chloroform. Parabens were visualized under ultravoiolet light. For quantitation, the image under ultraviolet light was captured digitally

and relative levels of bands were analysed by image analysis using the software packages Transform 3.4 (Fortner) and Origin 6.0.

Extraction of parabens from human breast tumour material and analysis by HPLCMS/MS

Samples of human breast tumour material (0.25 g) were chopped finely with a sterile razor and homogenized in a mixture of 6.25 ml of ethanol and 6.25 ml of acetone. This mixture was left with periodic shaking overnight in a sealed glass Corex tube. The next day, the mixture was spun at 2500 rpm for 10 min on a bench centrifuge at room temperature. The supernatant was removed to a clean Corex tube. The pellet was re-extracted with a further 1.5 ml of ethanol and 1.5 ml of acetone, spun and the two supernatants pooled. The total supernatant was dried under nitrogen at room temperature. To the residue was added 6 ml of 70% (v/v) aqueous methanol; the mixture was vortexed and then incubated overnight at −20 °C. The next day, the mixture was spun at 3200 rpm for 20 min at 4 °C and the supernatant was removed to a clean Corex tube. The pellet was re-extracted with a further 1 ml of 70% (v/v) aqueous methanol by vortexing and spun again at 3200 rpm for 20 min at 4 °C. The two supernatants were pooled and dried under nitrogen for analysis by HPLCMS/MS.

The extracts were dissolved in HPLC mobile phase (0.25 ml) and the paraben concentration determined by HPLCMS/MS. Samples (20 µl) of the final extracts were chromatographed on a Hypersil Elite C18 column (150 × 2.1 mm; 5 µm) at a flow rate of 0.3 ml min^{-1} and eluted with a linear binary gradient of 15 mM ammonium acetate pH 4.5 (A) and acetonitrile (B) (t = 0 min A 70%, t = 15 min A 40%, t = 16 min A 70%, t = 25 min next injection). The HPLC retention times for the paraben standards are provided in Table 1. The parabens were detected with a Sciex API 2000 triple quadrupole mass spectrometer equipped with a heated nebulizer probe operated in the negative ion mode. Optimal setting of the instrument for detection by mass reaction monitoring (MRM) was established empirically by infusion of paraben standards (1 µg ml^{-1}). The mass transitions selected for MRM detection utilized the fragmentation of the deprotonated molecular ion and are listed in Table 1. Chromatographic peaks corresponding to individual parabens were detected automatically and the mass of analyte calculated after interpolation from calibration curves prepared over the working range 1–300 ng ml^{-1} using the Analyst™ (PE Biosystems) software package.

Extractions were performed in groups such that each group of two to five tumour extractions had one blank extraction carried out alongside, with all procedures identical except for the omission of tumour material. However, analysis by HPLCMS/MS was carried out for all samples on the same day sequentially. Final paraben concentrations were calculated by subtraction of the values obtained from the corresponding blank extraction. Because the blank values showed variation, statistical analysis was performed using the paired t-test method (Snedecor & Cochran, 1980).

Table 1—Paraben standards: HPLC retention times and mass transition for MRM detection

Analyte	HPLC retention time (min)	Mass transition (Q1–Q3; m/z) for MRM detection
Methylparaben	4.6	151.1–92.1
Ethylparaben	7.3	165.1–92.1
n-Propylparaben	10.6	179.1–92.1
Isobutylparaben	13.4	193.1–92.1
n-Butylparaben	13.7	193.1–92.1
Benzylparaben	14.0	227.3–92.1

RESULTS

Extraction of parabens from breast tissue and detection by thin-layer chromatography

In initial exploratory experiments it was possible to detect parabens in human breast tissue using the extraction procedures described in the Materials and Methods section, followed by thin-layer chromatography against paraben standards. Aliquots (10–400 ng) of methylparaben, ethylparaben, n-propylparaben, n-butylparaben and isobutylparaben were run on thin-layer plates and could be detected under ultraviolet light. Under these conditions all the paraben standards ran to the same position, which was, on average, 0.47 ± 0.03 of the distance to the solvent front. Extracts of human breast tissue contained compounds visible under ultraviolet light at the same relative position as the paraben standards. From rough comparison by eye of the relative levels of paraben standards, it was estimated over six separate extractions that the samples contained in the region of 10–50 ng paraben per g breast tissue. Figure 1 shows the results of one experiment in which three aliquots (97, 194 and 388 ng) of n-butylparaben standards were run on thin-layer plates alongside the extract of 1 g of breast tissue. The relative intensities of the resulting bands under ultraviolet light were subjected to image analysis and plotted as a standard curve shown in Fig. 1. The relative intensity of the paraben band extracted from 1 g of tissue was 11 730, which corresponded to 47.1 ng paraben g^{-1} tissue.

It was on the basis of these preliminary results that we then proceeded to more detailed identification of individual parabens by HPLCMS/MS

Extraction of parabens from human breast tumours and analysis by HPLCMS/MS

Retention times and mass transition for MRM detection for the six paraben standards are shown in Table 1.

Parabens were extracted from a sample of each of 20 human breast tumours and extracts were analysed by HPLCMS/MS against paraben standards as described in the Materials and Methods section. Chromatographic peaks due to methylparaben, ethylparaben, n-propylparaben, n-butylparaben and isobutylparaben were seen in breast tumour extracts and were well resolved from one another. No peaks due to benzylparaben at its retention time of 14.0 min were seen in any of the tumour extracts. A typical chromatogram is shown in Fig. 2.

At a practical level, extractions were performed in small groups such that each group contained between two and five tumour samples together with one blank extraction. The blank extraction was performed with all procedures identical, except for the omission of tumour

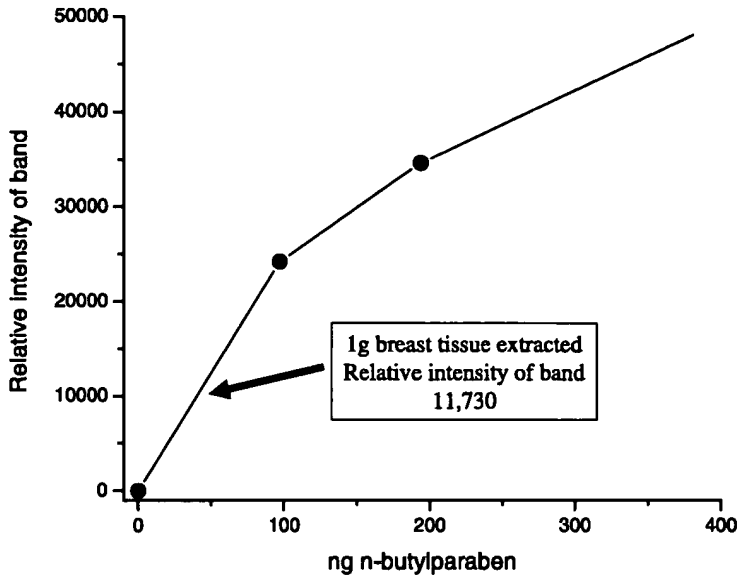


Figure 1. Detection of parabens from human breast tissue by thin-layer chromatography. Three aliquots of *n*-butylparaben (97, 194 and 388 ng) were run as standards on thin-layer plates alongside the extract of 1 g of breast tissue, and the relative intensities of the resulting bands under ultraviolet light were subjected to image analysis. The relative intensities of the bands for the three aliquots of *n*-butylparaben were plotted as a standard curve as shown. The relative intensity of the paraben band extracted from 1 g of tissue was 11 730, which calculated to an equivalent of 47.1 ng of paraben.

material. The concentrations of parabens in the 20 tumours as measured by HPLCMS/MS were corrected by subtraction of the corresponding blank value. Results are shown in Table 2. Because the blank values showed variation, the statistical significance of the mean corrected concentrations of each paraben in the 20 tumour extracts was tested by the paired *t*-test method, thus enabling the confidence limits of these mean values to be calculated (Table 3).

The reasons for the blank values for parabens, and their variation, are not clear. The MS data indicated that the blank values were genuinely parabens and not other contaminating compounds. The blank values did not come from the HPLCMS/MS procedure because blanks through the equipment were entirely negative. The blank values came from the extraction procedure itself. In a series of 30 blanks carried out on individual parts of the extraction procedure, it was not possible to identify any one specific reagent or procedure contributing to the blank value. However, when blank values were subtracted from the corresponding tumour extract values, 18/20 tumour extractions showed values of total paraben above the blank values. Values for total paraben present in the 20 tumour samples were 0–54.5 ng g^{-1} tissue, with an overall mean value of 20.6 ng g^{-1}. Methylparaben was present at the highest level, with an average value of 12.8 ± 2.2 ng g^{-1} tissue. This represented 62% of the total paraben recovered in the extraction. Benzylparaben was not detected in any tumour extract.

Estimates of recovery of parabens from the extraction procedure were made by spiking samples with benzylparaben, because this was the only paraben not detected in any blank or tumour extract. Analysis by HPLCMS/MS of three extraction blank samples, each spiked with 200 ng of benzylparaben, gave an average recovery of this paraben of 48.5% ± 4.8%.

DISCUSSION

Mean concentrations of each of six parabens in extracts of 20 human breast tumours (in the range 0–12.8 ng g^{-1} tissue; Table 3) have been measured with acceptable confidence. The reasons for the analytical blank values for parabens in these studies have not been identified definitively but probably relate to the ubiquitous use of parabens as preservatives even in laboratory detergents and personal care products of the operators. Analogous problems have been encountered with the measurement of phthalate esters because of their common use as plasticizers and their ubiquitous dispersal as impurities in solvents, water, glassware and many items of clinical and analytical laboratory equipment (Lopez-Aviva *et al.*, 1990; Leung & Giang, 1993; Colon *et al.*, 2000). More recent work in these laboratories (unpublished) has shown that immersion of all glassware in 1.0 M aqueous sodium hydroxide, followed by copious rinsing with double-distilled water, prior to use of this glassware in tissue extraction greatly reduces the blank values of paraben concentrations as measured by HPLCMS/MS. This addition to the analytical procedure is therefore recommended for use in further studies on paraben concentrations in tissues.

The total mean paraben level was found to be of the order of 20 ng g^{-1} tissue. This adds parabens to the list of environmental oestrogenic chemicals that can be found to accumulate in the human breast and already includes polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) (Dobson, 1993, Hardell *et al.*, 1996; Guttes *et al.*, 1998; Stellman *et al.*, 1998, 2000). Comparisons between the relative levels of parabens and other pollutants are not easy because several factors have to be

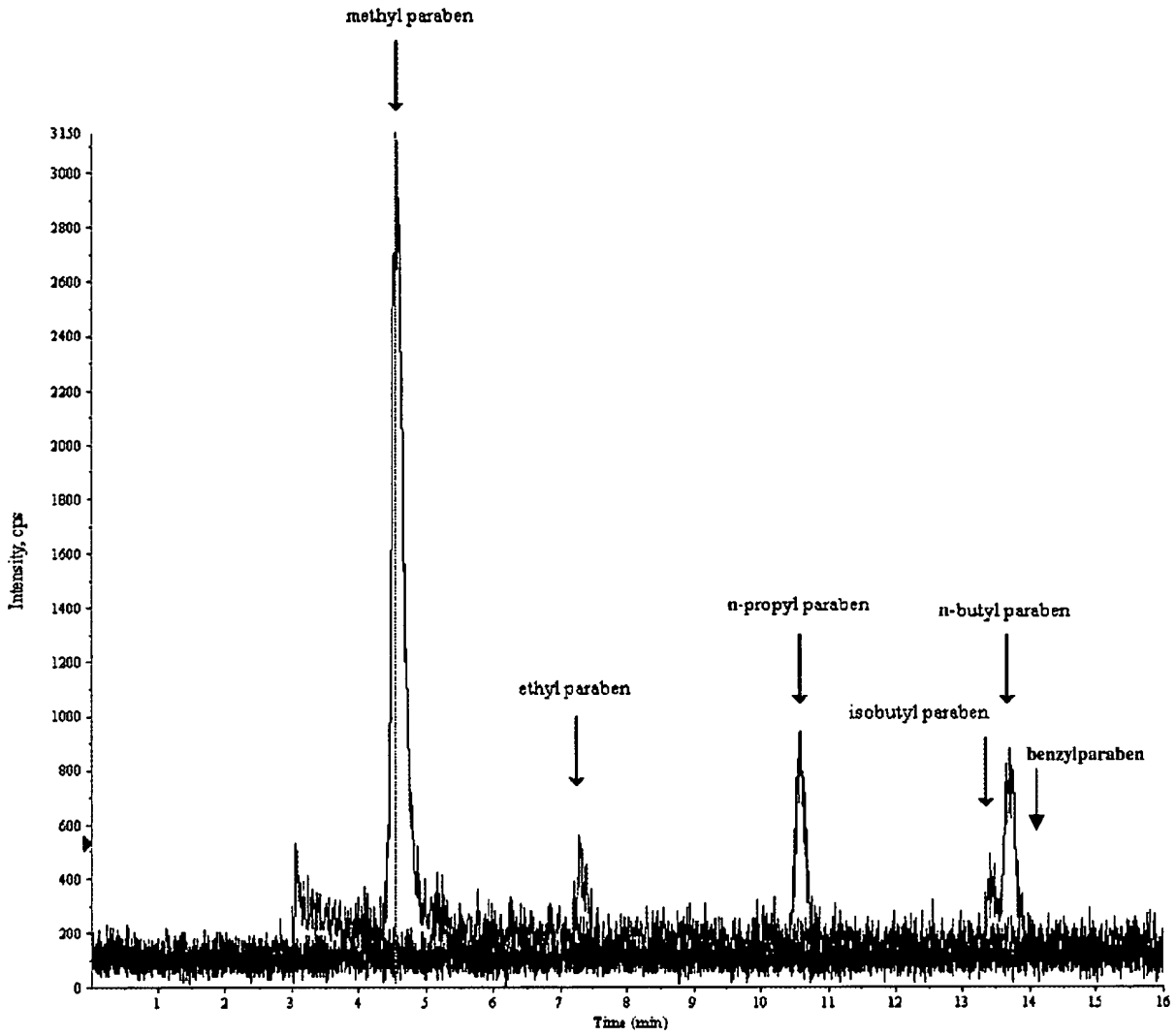


Figure 2. The HPLCMS/MS chromatograms for methylparaben, ethylparaben, *n*-propylparaben, isobutylparaben and *n*-butylparaben in a human breast tumour extract. Tumour tissue was extracted as described in the text, chromatographed on a Hypersil Elite HPLC column and detected by tandem mass spectrometry in the mass reaction monitoring mode. The annotated arrows indicate the identity of the peaks evident in the chromatograms. Benzylparaben was not seen.

taken into account, including the source of tissue used and the number of isomers or congeners. Most studies of bioaccumulation of pollutant chemicals are carried out by using serum or urine and studies using breast adipose tissue are few. Furthermore, for parabens there are only six commonly used forms whereas for PCBs there are 209 congeners. Studies of breast adipose tissue from women in Long Island, New York, without breast cancer showed a mean body burden for 14 PCB congeners of 267 ng g^{-1} and for seven OCPs of 707.5 ng g^{-1} (Stellman *et al.*, 1998). However, Table 4 shows that levels in breast tissue of individual pesticide residues and PCB congeners vary substantially. Although knowledge of total body burdens of these compounds is far from complete, the accumulation of parabens in breast tissue does fall within the broad range of these other compounds.

In the present study, paraben concentrations measured in tumours were unequivocally of the esters themselves. This demonstrates that at least a proportion of the parabens present in cosmetic, food and pharmaceutical products can be absorbed and retained in human body tissues without hydrolysis by tissue esterases to the common metabolite *p*-hydroxybenzoic acid. These results complement earlier studies in which there was evidence that the oestrogenic properties of these parabens in culture of human breast cancer cells were also due to the esters themselves and not to a common metabolite (Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003). However, these studies cannot identify either the source of the parabens or whether they entered the human body by an oral or by a topical route. Nor can they identify whether the parabens entered the human breast by a systemic route or through non-systemic mechanisms involving simply local absorption and diffusion from chemical overload of topical preparations applied to the breast area. Recent evaluation of parabens in uterotrophic assays has shown them to give oestrogenic responses in immature

Table 2—The HPLCMS/MS analysis of parabens in 20 human breast tumours[a]

Tumour extract	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**Mean**	**SEM**
Benzylparaben	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.0**	**0.0**
Isobutylparaben	5.2	0.1	5.2	3.3	2.8	3.5	2.5	1.9	4.9	2.2	1.7	3.9	2.2	2.3	1.2	2.0	1.7	0.0	1.0	0.0	**2.4**	**0.4**
n-Butylparaben	15.3	7.2	29.5	22.4	14.3	15.5	9.8	6.7	10.3	7.4	2.4	14.7	7.1	6.7	7.4	7.2	5.3	3.1	5.7	3.2	**10.1**	**1.5**
n-Propylparaben	6.5	7.9	18.6	18.4	10.9	17.9	10.0	10.5	11.6	6.9	9.1	16.5	10.2	17.2	10.1	15.4	4.2	5.3	5.5	5.6	**10.9**	**1.1**
Ethylparaben	7.2	3.0	9.6	6.3	4.6	2.3	1.9	1.8	7.0	3.2	1.8	6.4	2.1	4.5	1.1	2.2	1.8	0.7	2.0	2.1	**3.6**	**0.6**
Methylparaben	34.2	20.6	53.0	49.9	34.4	37.6	27.3	19.6	35.7	16.0	17.2	36.4	21.6	39.6	18.5	28.8	36.7	8.2	12.0	10.7	**27.9**	**2.8**
Total paraben	68.3	38.8	115.9	100.3	67.0	76.9	51.4	40.4	69.5	35.7	32.1	77.9	43.1	70.3	38.3	55.6	49.7	17.3	26.2	21.6	**54.8**	**5.8**
Blank value	**1**	**2 as 1**	**3**	**4 as 3**	**5 as 3**	**6**	**7 as 6**	**8 as 6**	**9 as 6**	**10**	**11 as 10**	**12 as 10**	**13 as 10**	**14 as 10**	**15**	**16 as 15**	**17 as 15**	**18**	**19 as 18**	**20 as 18**	**Mean**	**SEM**
Benzylparaben	0.0		0.0			0.0				0.0					0.0			0.0			**0.0**	**0.0**
Isobutylparaben	0.0		0.0			2.2				2.1					2.3			1.1			**1.3**	**0.4**
n-Butylparaben	6.5		18.0			9.3				4.0					8.2			2.2			**8.0**	**2.3**
n-Propylparaben	2.0		13.4			12.0				6.8					8.4			4.8			**7.9**	**1.8**
Ethylparaben	1.9		2.2			1.9				1.9					0.0			1.6			**1.6**	**0.3**
Methylparaben	10.1		27.8			20.5				10.3					11.6			9.9			**15.0**	**3.0**
Total paraben	20.5		61.4			45.9				25.0					30.6			19.6			**33.8**	**6.8**
Tumour less blank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**Mean**	**SEM**
Benzylparaben	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	**0.0**	**0.0**
Isobutylparaben	5.2	0.1	5.2	3.3	2.8	1.3	0.3	−0.3	2.7	0.2	−0.4	1.8	0.1	0.2	−1.1	−0.3	−0.6	−1.1	−0.1	−1.1	**0.9**	**0.4**
n-Butylparaben	8.8	0.7	11.5	4.4	−3.7	6.2	0.5	−2.6	1.0	3.4	−1.6	10.7	3.1	2.7	−0.8	−1.0	−2.9	1.0	3.5	1.0	**2.3**	**1.0**
n-Propylparaben	4.5	5.9	5.2	5.0	−2.5	5.9	−2.0	−1.5	−0.4	0.2	2.3	9.7	3.4	10.4	1.7	7.0	−4.3	0.5	0.7	0.8	**2.6**	**0.9**
Ethylparaben	5.3	1.2	7.4	4.1	2.4	0.5	0.0	−0.1	5.1	1.3	−0.2	4.5	0.2	2.6	1.1	2.1	1.8	−0.9	0.4	0.5	**2.0**	**0.5**
Methylparaben	24.1	10.5	25.2	22.1	6.6	17.1	6.8	−0.9	15.2	5.7	6.9	26.1	11.3	29.3	6.9	17.2	25.1	−1.8	2.1	0.8	**12.8**	**2.2**
Total paraben	47.9	18.4	54.5	38.9	5.6	31.0	5.5	−5.5	23.7	10.7	7.1	52.8	18.1	45.3	7.7	25.0	19.1	−2.3	6.6	2.0	**20.6**	**4.2**

[a] Paraben extractions were performed in small groups such that each group contained between two and five tumour samples together with one corresponding blank extraction. The blank extraction was performed with all procedures identical except for the omission of tumour material. Results are shown in ng g^{-1} tumour for the 20 extractions and for the corresponding blank values. The concentrations of parabens in the 20 tumours were then each corrected by subtraction of the corresponding blank value.

Table 3—Confidence limits of mean concentrations (ng g^{-1}) of parabens in the 20 human breast tumours of Table 2

Tumour minus blank	Mean	Confidence limit
Benzylparaben	0.0	0.0–0.0 (95%)
Isobutylparaben	0.9	0.1–1.7 (90%)
n-Butylparaben	2.3	0.3–4.3 (95%)
n-Propylparaben	2.6	0.7–4.5 (95%)
Ethylparaben	2.0	1.0–3.0 (95%)
Methylparaben	12.8	8.2–17.4 (95%)
Total paraben	20.6	11.8–29.4 (95%)

rodent uterus only when administered subcutaneously or topically but not orally (Routledge *et al.*, 1998; Hossaini *et al.*, 2000; Darbre *et al.*, 2002, 2003), which suggests that skin penetration may be an important route for entry to the body.

A major issue in studies of accumulation of environmental pollutants in body tissues is whether the levels reached could be sufficiently high to exert any biological action. In four of the 20 tumours, total paraben concentration was more than twice the average level and, allowing for a 50% recovery of parabens through the analytical procedure, the corrected average level of parabens was ca. 100 ng g^{-1} tissue. This concentration may be compared with the level (ca. 150 ng ml^{-1}; 10^{-6} M) in culture medium at which *n*-propylparaben, *n*-butylparaben and isobutylparaben stimulated growth of oestrogen-dependent MCF7 human breast cancer cells (Okubo *et al.*, 2001; Byford *et al.*, 2002; Darbre *et al.*, 2002, 2003). It is therefore not inconceivable that the levels of parabens measured in this study could exert oestrogenic effects on epithelial cells in the human breast. Although in rodent uterotrophic assays the levels of parabens were administered at a higher range of 0.1–10 mg g^{-1} body weight (Routledge *et al.*, 1998, Darbre *et al.*, 2002, 2003), these studies did not incorporate any measurements of paraben levels reached in the uterus at the time of response, which prevents assessment of the concentrations needed for physiological response.

It is interesting that the paraben detected in greatest amounts was methylparaben. This may reflect the more widespread use of methylparaben in consumer products (Rastogi *et al.*, 1995). Alternatively, it may reflect the greater ability of methylparaben to be absorbed into body tissues and to resist hydrolysis by esterases of human skin and subcutaneous fat tissue (Lobemeier *et al.*, 1996). By contrast, benzylparaben was not found in any of the 20 breast tumours and this may similarly be attributed to its less frequent use in consumer products.

These measurements of paraben concentrations in breast tumours open the way technically to more detailed determinations of paraben levels in human body tissues. This study used 20 breast tumour samples because of the availability of the material. However, it will now be important to measure levels in corresponding normal tissue to determine whether there is any difference between normal and cancer tissues. Larger studies also are needed to give more representative values for body burdens in different tissues and across the human population. A main problem with human breast tumour samples is the varied infiltration of the tumour with fatty tissue and blood vessels and it will be important in future work therefore to have more precise histological information on the tumours in order especially to be able to relate results to fatty versus non-fatty tissue. It would be informative to ascertain whether there are any gradients in the accumulation of

Table 4—Summary of mean concentrations (ng g^{-1}) of individual pesticide residues and PCB congeners in human breast adipose tissue from control and breast cancer patients from two published studies

Pesticide or PCB	Control	Breast cancer	Control	Breast cancer
HCB	206	343	16	18
βHCH	72	84	16	20
Oxychlordane			39	46
trans-Nonachlor			40	51
p,p'-DDE	450	838	374	419
o,p'-DDD			13	16
p,p'-DDT	24	30	12	12
PCB 74			27	30
PCB 99			14	19
PCB 118	58	85	24	30
PCB 138	176	241	22	29
PCB 146			7	9
PCB 153	437	664	63	76
PCB 156	61	64	9	11
PCB 167			1	2
PCB 170	229	259	11	14
PCB 172			2	2
PCB 178			3	4
PCB 180	258	400	34	42
PCB 183			4	6
PCB 187			13	16
Reference	Guttes *et al.* (1998)	Guttes *et al.* (1998)	Stellman *et al.* (2000)	Stellman *et al.* (2000)
Location	Hesse, Germany	Hesse, Germany	New York, USA	New York, USA
Number of samples	*n* = 20	*n* = 45	*n* = 323	*n* = 232

parabens across the human breast from axilla to sternum in case the topical application of cosmetic at one place influences the levels of parabens detectable. It will also be important to know whether there is any difference between levels detectable in breast tumours compared with adjacent non-tumour material in order to determine whether higher levels of paraben accumulation might be present in the tumours. Such information, taken together with that of concentrations in tissues of endogenous steroid hormones and other xenoestrogens, should enable assessment to be made of the impact of these weakly oestrogenic parabens on human health, and whether paraben accumulation from currently permitted levels in cosmetics, foods and pharmaceuticals remains acceptable.

Acknowledgements

We are grateful for financial support from the Seedcorn Fund of the Veterinary Laboratories Agency (P.D.D., N.G.S., M.J.S.) and for statistical advice from Dr M. C. Denham, School of Applied Statistics, University of Reading.

REFERENCES

Bairati C, Goi G, Lombardo A, Tettamanti G. 1994. The esters of *p*-hydroxy-benzoate (parabens) inhibit the release of lysosomal enzymes by mitogen-stimulated peripheral human lymphocytes in culture. *Clin. Chim. Acta* **224**: 147–157.

Bando H, Mohri S, Yamashita F, Takakura y, Hashida M. 1997. Effects of skin metabolism on percutaneous penetration of lipophilic drugs. *J Pharm. Sci.* **86**: 759–761.

Blair RM, Fang H, Branham WS, Hass BS, Dial SL, Moland CL, Tong W, Shi L, Perkins R, Sheehan DM. 2000. The estrogen receptor binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci.* **54**: 138–153.

Byford JR, Shaw LE, Drew MGB, Pope GS, Sauer MJ, Darbre PD. 2002. Oestrogenic activity of parabens in MCF7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **80**: 49–60.

Colon I, Caro D, Bourdony CJ, Rosario O. 2000. Identification of phthalate esters in the serum of young Puerto Rican girls with premature breast development. *Environ. Health Perspect.* **108**: 895–900.

Darbre PD. 1998. Environmental contaminants in milk: the problem of organochlorine xenobiotics. *Biochem. Soc. Trans.* **26**: 106–112.

Darbre PD. 2001. Hypothesis: underarm cosmetics are a cause of breast cancer. *Eur. J. Cancer Prevent.* **10**: 389–393.

Darbre PD. 2003. Underarm cosmetics and breast cancer. *J. Appl. Toxicol.* **23**: 89–95.

Darbre PD, Byford JR, Shaw LE, Horton RA, Pope GS, Sauer MJ. 2002. Oestrogenic activity of isobutylparaben *in vitro* and *in vivo*. *J Appl. Toxicol.* **22**: 219–226.

Darbre PD, Byford JR, Shaw LE, Hall S, Coldham NG, Pope GS, Sauer MJ. 2003. Oestrogenic activity of benzylparaben. *J Appl. Toxicol.* **23**: 43–51.

Derache R, Gourdon J. 1963. Metabolism of a food preservative: *p*-hydroxybenzoic acid and its esters. *Food Cosmet. Toxicol.* **1**: 189–195.

Dobson S, Gilbert M, Howe PD. 1989. DDT and its derivatives — environmental aspects. *Environ. Health Crit.* **83**: 1–79.

Dobson S. 1993. Polychlorinated biphenyls and terphenyls. *Environ. Health Crit.* **140**: 1–496.

Elder RL. 1984. Final report on the safety assessment of methylparaben, ethylparaben, propylparaben and butylparaben. *J. Ame. Coll. Toxicol.* **3**: 147–209.

Fang H, Tong W, Shi LM, Blair R, Perkins R, Branham W, Hass BS, Xie Q, Dial SL, Moland CL, Sheehan DM. 2001. Structure–activity relationships for a large diverse set of natural, synthetic, and environmental estrogens. *Chem. Res. Toxicol.* **14**: 280–294.

Fischmeister I, Hellgren L, Vincent J. 1975. Infrared spectroscopy for tracing of topically applied ointment vehicles and active substances on healthy skin. *Arch. Dermatol. Res.* **253**: 63–69.

Guttes S, Failing K, Neumann K, Kleinstein J, Georgii S, Brunn H. 1998. Chlororganic pesticides and polychlorinated biphenyls in breast tissue of women with benign and malignant breast disease. *Arch. Environ. Contam. Toxicol.* **35**: 140–147.

Hardell L, Lindstrom G, Liljegren G, Dahl P, Magnuson A. 1996. Increased concentrations of octachlorodibenzo-p-dioxin in cases with breast cancer — results from a case-control study. *Eur. J Cancer Prevent.* **5**: 351–357.

Harvey PW. 2003. Parabens, oestrogenicity, underarm cosmetics and breast cancer: a perspective on a hypothesis (Editorial) *J. Appl. Toxicol.* **23**: 285–288.

Heim F, Leuschner F, Wunderlich G. 1957. Metabolism of *p*-hydroxybenzoic acid ethyl ester. *Klin. Wochenschr.* **35**: 823–825.

Hossaini A, Larsen JJ, Larsen JC. 2000. Lack of oestrogenic effects of food preservatives (parabens) in uterotropic assays. *Food Chem. Toxicol.* **38**: 319–323.

Ishidate M, Hayashi M, Sawada M, Matsuoka A. 1978. Cytotoxicity test on medical drugs. Chromosome aberration tests with Chinese hamster cells *in vitro*. *Eisei Shikensho Hokoku* **96**: 55–61.

Jin-Sung P, Lee BJ, Kang KS, Tai JH, Cho JJ, ChoMH, Inoue T, Lee YS. 2000. Hormonal effects of several chemicals in recombinant yeast, MCF7 cells and uterotropic assays in mice. *Microbiol. Biotechnol.* **10**: 293–299.

Jones PS, Thigpen D, Morrison JL, Richardson AP. 1956. *p*-Hydroxybenzoic acid esters as preservatives. III. The physiological disposition of *p*-hydroxybenzoic acid and its esters. *J Am. Pharm. Assoc. Sci. Ed.* **45**: 265–273.

Kitigawa S, Li H, Sato S. 1997. Skin permeation of parabens in excised guinea pig dorsal skin, its modification by penetration enhancers and their relationship with *n*-octanol/water partition coefficients. *Chem. Pharm. Bull. (Tokyo)* **45**: 1354–1357.

Kiwada H, Awazu S, Hanano M. 1979. The study on the biological fate of paraben at the dose of practical usage in rat. I. The metabolism and excretion of ethyl-*p*-hydroxybenzoate (ethyl paraben) and *p*-hydroxybenzoic acid. *J. Pharmacobio-Dyn.* **2**: 356–364.

Komatsu H, Suzuki M. 1979. Percutaneous absorption of butylparaben through guinea pig skin *in vitro*. *J. Pharm. Sci.* **68**: 596–598.

Leung S, Giang BY. 1993. Phthalate esters as potential contaminants during GC analysis of environmental samples using electron capture and mass spectrometric detectors. *Bull. Environ. Contam. Toxicol.* **50**: 528–532.

Lipworth L. 1995. Epidemiology of breast cancer. *Eur. J. Cancer Prevent.* **4**: 7–30.

Lobemeier C, Tschoetschel C, Westie S, Heymann E. 1996. Hydrolysis of parabenes by extracts from differing layers of human skin. *Biol. Chem.* **377**: 647–651.

Lopez-Aviva V, Milanes J, Constantine F, Beckert WF. 1990. Typical phthalate ester contamination incurred using EPA method 8060. *J. Assoc. Off. Anal. Chem.* **73**: 709–720.

Mason MM, Cate CC, Baker J. 1971. Toxicology and carcinogenesis of various chemicals used in the preparation of vaccines. *Clin. Toxicol.* **4**: 185–204.

Matsuoka A, Hayashi M, Ishidate M. 1979. Chromosomal aberration tests on 29 chemicals combined with S9 mix *in vitro*. *Mutat. Res.* **66**: 277–290.

Miller WR. 1996. *Estrogen and Breast Cancer*. Chapman and Hall: London.

Murrell WG, Vincent JM. 1950. The 4-hydroxybenzoic acid esters and related compounds. 4. The bacteriostatic action of 4-hydrozybenzoic acid *n*-alkyl esters. *J. Soc. Chem. Ind.* **69**: 109–113.

Nakagawa Y, Maldeus P. 1998. Mechanism of *p*-hydroxybenzoate ester-induced mitochondrial dysfunction and cytotoxicity in isolated rat hepatocytes. *Biochem. Pharmacol.* **55**: 1907–1914.

Nishihara T, Nishikawa JI, Kanayama T, Dakeyama F, Saito K, Imagawa M, Takatori S, Kitagawa Y, Hori S, Utsumi H. 2000.

Estrogenic activities of 517 chemicals by yeast two-hybrid assay. *J. Health Sci.* **46**: 282–298.

Oishi S. 2001. Effects of butylparaben on the male reproductive system in rats. *Toxicol. Ind. Health* **17**: 31–39.

Okubo T, Yokoyama Y, Kano K, Kano I. 2001. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ERα and PR. *Food Chem. Toxicol.* **39**: 1225–1232.

Pedersen KL, Pedersen SN, Christiansen LB, Korsgaard B, Bjerregaard P. 2000. The preservatives ethyl-, propyl-, and butylparaben are oestrogenic in an *in vivo* fish assay. *Pharmacol. Toxicol.* **86**: 110–113.

Phillips JC, Topp CS, Gangolli SD. 1978. The metabolism of ethyl and *n*-propyl-*p*-benzoate ('parabens') in male cats. *Toxicol. Lett.* **2**: 137–142.

Pope GS, Cunningham JM, Jenkins N, Waites GMH, Watts GE. 1990. Oestradiol-17β in testis and jugular venous plasma of intact and hemi-castrated prepubertal lambs and in jugular venous plasma of castrated prepubertal lambs. *Anim. Reprod. Sci.* **22**: 9–19.

Rastogi SC, Schouten A, De Kruijf N, Weijland JW. 1995. Contents of methyl-, ethyl-, propyl-, butyl- and benzylparaben in cosmetic products. *Contact Dermat.* **32**: 28–30.

Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. 1998. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **153**: 12–19.

Snedecor GW, Cochran WG. 1980. *Statistical Methods* (7th edn). Iowa State University Press: Iowa.

Sonawane BR. 1995. Chemical contaminants in human milk: an overview. *Environ. Health Perspect.* **103**: 197–205.

Stellman SD, Djordjevic MV, Muscat JE, Gong L, Bernstein D, Citron ML, White A, Kemeny M, Busch E, Nafziger AN. 1998. Relative abundance of organochlorine pesticides and polychlorinated biphenyls in adipose tissue and serum of women in Long Island, New York. *Cancer Epidemiol. Biomarkers Prevent.* **7**: 489–496.

Stellman SD, Djordjevic MV, Britton JA, Muscat JE, Citron ML, Kemeny M, Busch E, Gong L. 2000. Breast cancer risk in relation to adipose concentrations of organochlorine pesticides and polychlorinated biphenyls in Long Island, New York. *Cancer Epidemiol. Biomarkers Prevent.* **9**: 1241–1249.

Tsukamoto H, Terada S. 1960. Metabolism of drugs. XXIII. Metabolic fate of *p*-hydroxybenzoic acid and its derivatives in rabbits. *Chem. Pharm. Bull. (Tokyo)* **8**: 1066–1070.

Tsukamoto H, Terada S. 1962. Metabolism of drugs. XXVI. Metabolic fate of *p*-hydroxybenzoic acid and its derivatives in rabbits. *Chem. Pharm. Bull. (Tokyo)* **10**: 86–90.

Tsukamoto H, Terada S. 1964. Metabolism of drugs. XLVII. Metabolic fate of *p*-hydroxybenzoic acid and its derivatives in rabbits. *Chem. Pharm. Bull. (Tokyo)* **12**: 765–769.

Whitworth CW, Jun HW. 1973. Influence of polysorbate 20 and sodium cholate on uptake of *p*-hydroxybenzoates by the frog Rana pipiens. *J. Pharm. Sci.* **62**: 1890–1891.

JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. **24**, 1–4 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jat.957

EDITORIAL

Significance of the Detection of Esters of *p*-Hydroxybenzoic Acid (Parabens) in Human Breast Tumours

Philip W. Harvey* and David J. Everett
Department of Toxicology, Covance Laboratories Ltd, Otley Road, Harrogate, North Yorkshire HG3 1PY, UK

Key words: breast cancer; hydroxybenzoic acid; parabens; oestrogen; tumour; carcinogenesis; underarm; deodorant; antiperspirant.

This issue of *Journal of Applied Toxicology* publishes the paper *Concentrations of Parabens in Human Breast Tumours* by Darbre *et al.* (2004), which reports that esters of *p*-hydroxybenzoic acid (parabens) can be detected in samples of tissue from human breast tumours. Breast tumour samples were supplied from 20 patients, in collaboration with the Edinburgh Breast Unit Research Group, and analysed by high-pressure liquid chromatography and tandem mass spectrometry. The parabens are used as antimicrobial preservatives in underarm deodorants and antiperspirants and in a wide range of other consumer products. The parabens also have inherent oestrogenic and other hormone related activity (increased progesterone receptor gene expression). As oestrogen is a major aetiological factor in the growth and development of the majority of human breast cancers, it has been previously suggested by Darbre that parabens and other chemicals in underarm cosmetics *may* contribute to the rising incidence of breast cancer. The significance of the finding of parabens in tumour samples is discussed here in terms of 1) Darbre *et al*'s study design, 2) what can be inferred from this type of data (and what can not, such as the cause of these tumours), 3) the toxicology of these compounds and 4) the limitations of the existing toxicology database and the need to consider data that is appropriate to human exposures.

INTRODUCTION

This issue of *Journal of Applied Toxicology* publishes the paper 'Concentrations of Parabens in Human Breast Tumours' by Darbre *et al.* (2004) which reports that esters of *p*-hydroxybenzoic acid (parabens) can be detected in samples of tissue from human breast tumours. Breast tumour samples were supplied from 20 patients in a collaboration with the Edinburgh Breast Unit Research Group, and analysed by high pressure liquid chromatography and tandem mass spectrometry. The parabens are used as antimicrobial preservatives in underarm deodorants and antiperspirants, and in a wide range of other consumer products. The parabens also have inherent oestrogenic activity (briefly reviewed in the next section) and oestrogen is a *major* aetiological factor in the growth and development of human breast cancer. It has previously been suggested that chemicals in underarm cosmetics may contribute to the rising incidence of breast cancer (Darbre, 2001; 2003; and see Harvey, 2003) and the significance of the finding of parabens in tumour samples is therefore highly topical.

* Correspondence to: Dr. P. W. Harvey, Department of Toxicology, Covance Laboratories Ltd, Otley Road, Harrogate, North Yorkshire HG3 1PY, UK.
E-mail: philip.harvey@covance.com

OESTROGEN AS A COMMON FACTOR IN BREAST CANCER AND PARABEN TOXICITY

It has been known for many years that oestrogen is the major aetiological factor in the development of breast cancer and, indeed, modern therapies continue to use pharmacological receptor blockade and synthetic suppression (e.g. aromatase inhibition) in clinical treatments (McPherson *et al.*, 1994; Wiseman, 1994; Elledge & Osbourne, 1997; Walker, 1999; Lønning, 2001; Beral *et al.*, 2003). Given this, it is logical to suggest that application of oestrogenic agents to areas adjacent to the breast may be an unnecessary risk in some women (in this context it has been suggested that first-degree relatives of breast cancer patients and peri-adolescent females would be at most risk of continued exposure to oestrogenic chemicals). The ubiquitous use of underarm deodorants and antiperspirants throughout the Western world means that millions of women have applied a range of chemicals to the axilla of the arm and it is surprising that only recently have some of these chemical ingredients been screened for the toxicologically important endpoints of inherent oestrogenic and hormonal activity.

There are now numerous reports that various parabens are oestrogenic. Lemini *et al.* (1997) reported that subcutaneous administration of *p*-hydroxybenzoic acid produced vaginal cornification and increased uterine weights (both classic effects of the action of endogenous oestradiol)

Received 30 September 2003
Revised 7 October 2003
Accepted 7 October 2003

in mice. Routledge *et al.* (1998) reported that butylparaben competed with [^{3}H]oestradiol in an oestrogen receptor binding assay, that methyl-, ethyl-, propyl- and butylparaben were weakly positive in a yeast oestrogen assay and that butylparaben was positive in an immature rat uterotrophic assay by the subcutaneous (but not oral; see later) administration route. In human MCF7 breast cancer cells, Byford *et al.* (2002) have shown that methyl-, ethyl-, *n*-propyl- and *n*-butylparaben are oestrogenic. Okubo *et al.* (2001) reported similar findings with ethyl-, propyl-, butyl-, isopropyl- and isobutylparaben and also that butylparaben and isobutylparaben increased progesterone receptor gene expression. Interestingly, oestrogen–progestagen hormone replacement therapy confers the greatest risk of breast cancer (Beral *et al.*, 2003) and the parabens show activity to both oestrogen and progesterone receptors. Darbre *et al.* (2002, 2003), using both MCF7 and ZR-75-1 human breast cancer cell lines, report oestrogenic activity for isobutylparaben and benzylparaben. These latter studies also reported oestrogenic activity *in vivo*: isobutylparaben resulted in a uterotrophic response in immature mice following subcutaneous administration but, of most significance, benzylparaben induced a uterotrophic response following *topical* administration (application to dorsal skin of 33 mg per mouse per day for 3 days; Darbre *et al.*, 2003). Parabens also have structures predicted to bind to the oestrogen receptor (Hong *et al.*, 2002).

Harvey (2003) provides a perspective on the dose levels reported to produce effects in short-term *in vivo* animal studies (i.e. while dose levels are relatively high, convention dictates that risk assessments would apply safety factors of at least 100-fold to data from animal studies when extrapolating to safe human exposures) and the relative lack of activity by the oral route (presumably due to metabolic breakdown, an effect that apparently does not occur with the more direct subcutaneous or topical administration routes in animal studies) as factors particularly relevant to risk assessments specific for cosmetic use, and the possibility of inappropriate extrapolation from the existing parabens animal toxicology database. In reviewing the various reports of paraben oestrogenicity, potency ranges from 500-fold less than oestradiol (reported by Lemini *et al.* (1997) in a rat uterotrophic assay following subcutaneous administration of *p*-hydroxybenzoic acid) to 10 000-fold less potent as reported by Routledge *et al.* (1998) for butylparaben in the *in vitro* yeast assay. Clearly there is a need to place human exposures of the parabens into perspective: contributions to the total body burden of oestrogenic agents include endogenous oestrogen and a variety of xenobiotics (e.g., resorcylic acid lactone residues in food; Everett *et al.*, 1987). Parabens represent just one class of these oestrogenic materials, all of which need to be considered both in terms of inherent oestrogenic potency as well as their actual concentrations in human tissues. Although oestrogenic potency of the parabens is relatively weak, the use patterns of underarm cosmetics and parabens in other products can result in long-term exposures.

SIGNIFICANCE OF THE DETECTION OF PARABENS IN BREAST TUMOURS

Darbre *et al.* (2004) have shown that a common group of chemicals used in underarm deodorant and antiperspirant formulations and other consumer products, previously generally regarded as safe but recently shown to possess oestrogenic activity in a wide variety of assays, can be detected in human breast tumour tissue. This finding would logically be a significant prerequisite criterion to the hypothesis that these compounds may be involved in, or in some way contribute to, the incidence of breast cancer (which has steadily risen over recent decades in the UK and elsewhere, paralleling, for example, underarm cosmetic usage; see Darbre, 2003) in that there would obviously need to be cellular exposure to these chemicals in order to induce any direct carcinogenic response. *If* the source of these chemicals was prior historical use of underarm cosmetics containing these ingredients (at present it is not known what the half-life or clearance of these chemicals from human breast tissue is, or the contribution from sources other than underarm cosmetics — see below), then such data suggest that these chemicals can be absorbed dermally and probably persist in human breast tissue. Several points must be made in discussing Darbre *et al.*'s (2004) findings:

(i) The detection of parabens in breast tumour tissue should not be taken to imply causality of the individual cancer, because the findings are essentially coincidental in nature.

(ii) 'Normal' breast tissue, and other tissue, was not analysed. Although the question remains of what levels occur in such control tissue, it should be recognized that apparently normal tissue at the *time* of biopsy may later develop a tumour (this is important because cancer represents a risk over a lifetime and not a single time point) and there are questions of what would be an appropriate control for this type of data.

(iii) The obvious route of entry into the breast tissue is local absorption from the underarm (because esters were detected rather than metabolites) and the source is probably therefore underarm cosmetics. However, the source needs to be confirmed and Darbre *et al.* make it clear that their study does not identify route.

(iv) Although the data could be consistent with local absorption, it would be interesting to establish what the levels of parabens are in other tissues (e.g. blood, adipose and those also sensitive to oestrogen).

(v) It is obvious that extraneous synthetic organic chemicals serve no useful function in the human breast but, the question is, have they caused harm?

(vi) Related to this, Darbre *et al.*'s (2004) study analysed parabens because of interest in their use in underarm cosmetics: other chemicals also may be present (because these types of study are records of single time points, the levels of a variety of extraneous chemicals could increase or decline over a lifetime).

(vii) Darbre *et al.*'s (2004) study shows the presence of parabens in breast tumour tissue: although it has been emphasized that the significance of this should not be overinterpreted, their route of disposition and possible effects on the breast are worthy of further investigation.

(viii) In the general context of the hypothesis, any response of cells in the breast will depend on the properties of the chemicals, the timing and relative duration of exposure (consider potential differences of effect

between adolescent exposure with the developing breast and exposure in later life), the dose and the interaction with other genetic and environmental factors.

GENERAL CONSIDERATIONS AND CONCLUSION

The findings of parabens in tumour samples are additional results in line with the general hypothesis that there may be a link between oestrogenic compounds commonly used in underarm cosmetics and other consumer products and breast cancer. The results alone, however, do not suggest that these chemicals caused the tumours in these patients. Darbre *et al.*'s findings invite several questions: how did the parabens get into the breast, are they persistent and could they do harm? The answers require further research.

The hypothesis that underarm cosmetics may contribute to the incidence of breast cancer has obvious implications, not least because of the size of the population potentially exposed. The role of oestrogen in breast cancer is clear. It is also now clear that the parabens are weakly oestrogenic and thus there is logic to the hypothesis when combined with other lines of evidence (Darbre, 2003). However, apparently little is known of any side-effects associated with long-term, low-level exposures to synthetic xenoestrogens. The use of underarm cosmetics presents a special case because of the direct application of the compounds to skin. Darbre *et al.*'s (2004) study indicates that paraben esters are detectable in breast tumour tissue, which could feasibly result from a previous history of cosmetic use, local dermal absorption and some degree of residue persistence, but the route also could be from other sources, such as orally if there was no metabolic transformation of the parent compound.

The hypothesis forwarded that underarm cosmetics may be implicated in the incidence of breast cancer (Darbre, 2003) has been discussed also in terms of the potential toxicity of oestrogenic formulation ingredients (Harvey, 2003). Although recent efforts have been made to examine 'antiperspirant use and the risk of breast cancer' (see Mirick *et al.* (2002), who report no association based on retrospective interview), there is a need for research that carefully focuses on chemical toxicity issues (i.e. the specific formulation ingredients and not simply underarm cosmetics *per se*). Research also should consider sensitive population subgroups (especially adolescents and firstdegree relatives of breast cancer patients) and requires designs with the sensitivity to elucidate any effects of long-term, low-level exposures to mixtures. As far as toxicological reviews and risk assessments of the parabens are concerned, they apparently have not taken into account recent evidence of inherent oestrogenic and hormonal activities (Soni *et al.*, 2002; Willis, 1995) and there is a perceived need to conduct up-to-date risk assessments on the suitability of each type of paraben specifically for their use in underarm cosmetics. Finally, Darbre *et al.*'s (2004) study is a contribution to a body of literature that reports chemicals in human breast tissue, with the suggestion that these compounds may be carcinogenic (Falck *et al.*, 1992; Snedeker, 2001), particularly breast organochlorine concentrations correlated with increased cancer risk (Aronson *et al.*, 2000) and related to oestrogenicity (Starek, 2003). Whether underarm cosmetics will prove to be a special case because of their direct application or not, unlike diffuse environmental exposures, individual use is preventable and the removal of oestrogenic formulants would effectively resolve at least one potential mechanistic factor central to this hypothesis.

REFERENCES

Aronson KJ, Miller AB, Woolcott CG, Sterns EE, McCready DR, Lickley LA, Fish EB, Hiracki GY, Holloway C, Ross T, Hanna WM, SenGupta SK, Weber JP. 2000. Breast adipose tissue concentrations of polychlorinated biphenyls and other organochlorines and breast cancer risk. *Cancer Epidemiol. Biomarkers Prev.* **9**: 55–63.

Beral V, Million Women Study Collaborators. 2003. Breast cancer and hormone replacement in the million women study. *Lancet* **362**: 419–427.

Byford JR, Shaw LE, Drew MG, Pope GS, Sauer MJ, Darbre PD. 2002. Oestrogenic activity of parabens in MCF7 human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **80**: 49–60.

Darbre PD. 2001. Underarm cosmetics are a cause of breast cancer. *Eur. J. Cancer Prev.* **10**: 389–393.

Darbre PD. 2003. Underarm cosmetics and breast cancer. *J. Appl. Toxicol.* **23**: 89–95.

Darbre PD, Byford JR, Shaw LE, Horton RA, Pope GS, Sauer MJ. 2002. Oestrogenic activity of isobutylparaben *in vitro* and *in vivo*. *J. Appl. Toxicol.* **22**: 219–226.

Darbre PD, Byford JR, Shaw LE, Hall S, Coldham NG, Pope GS, Sauer MJ. 2003. Oestrogenic activity of benzylparaben. *J. Appl. Toxicol.* **23**: 43–51.

Darbre PD, Aljarrah A, Miller WR, Coldham NG, Sauer MJ Pope GS. 2004. Concentrations of parabens in human breast tumours. *J. Appl. Toxicol.* **24**: 5–13.

Elledge RM, Osbourne CK. 1997. Oestrogen receptors and breast cancer (Editorial). *Br. Med. J.* **314**: 1834.

Everett DJ, Perry CJ, Scott KA, Martin BW, Terry MK. 1987. Estrogenic potencies of resorcylic acid lactones and 17β-estradiol in female rats. *J. Toxicol. Environ. Health* **20**: 435–443.

Falck F, Ricci A, Wolff MS, Godbold J, Deckers P. 1992. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. *Arch. Environ. Health* **47**: 143–146.

Harvey PW. 2003. Parabens, oestrogenicity, underarm cosmetics and breast cancer: a perspective on a hypothesis. *J. Appl. Toxicol.* **23**: 285–288.

Hong H, Tong W, Fang H, Shi L, Xie Q, Perkins R, Walker JD, Branham W, Sheehan DM. 2002. Prediction of estrogen receptor binding for 58 000 chemicals using an integrated system of a tree based model with structural alerts. *Environ. Health Perspect.* **110**: 29–36.

Lemini C, Silva G, Timossi C, Luque D, Valverde A, Gonzalez-Marti M, Hernandez A, Rubio-Poo C, Chavez Lara B, Valezuela F. 1997. Estrogenic effects of *p*-hydroxybenzoic acid in CD1 mice. *Environ. Res.* **75**: 130–134.

Lønning PE. 2001. Aromatase inhibitors and inactivators in breast cancer (Editorial). *Br. Med. J.* **323**: 880–881.

McPherson KM, Steel CM, Dixon JM. 1994. ABC of breast diseases: Breast cancer-epidemiology, risk factors and genetics. *Br. Med. J.* **309**: 1003–1006.

Mirick DK, Davis S, Thomas DB. 2002. Antiperspirant use and the risk of breast cancer. *J. Natl. Cancer Inst.* **94**: 1578–1580.

Okubo T, Yokoyama Y, Kano K, Kano I. 2001. ER-dependent estrogenic activity of parabens assessed by proliferation of human breast cancer MCF-7 cells and expression of ER alpha and PR. *Food Chem. Toxicol.* **39**: 1225–1232.

Routledge EJ, Parker J, Odum J, Ashby J, Sumpter JP. 1998. Some alkyl hydroxy benzoate preservatives (parabens) are estrogenic. *Toxicol. Appl. Pharmacol.* **153**: 12–19.

Snedeker SM. 2001. Pesticides and breast cancer risk: a review of DDT, DDE and dieldrin. *Environ. Health Perspect.* **109** (Suppl. 1): 35–47.

Soni MG, Taylor SL, Greenberg NA, Burdock GA. 2002. Evaluation of the health effects of methyl paraben: a review of the published literature. *Food Chem. Toxicol.* **40**: 1335–1373.

Starek A. 2003. Estrogens and organochlorine xenoestrogens and breast cancer risk. *Int. J. Occup. Med. Environ. Health* **16**: 113–124.

Walker RA. 1999. Hormonal mechanisms in breast cancer. In *Endocrine and Hormonal Toxicology*, Harvey PW, Rush KC, Cockburn A (eds). John Wiley: Chichester; 487–506.

Willis L. 1995. Final report on the safety assessment of isobutylparaben and isopropylparaben. *J. Am. Coll. Toxicol.* **14**: 364–372.

Wiseman H. 1994. *Tamoxifen. Molecular Basis of Use in Cancer Treatment and Prevention.* John Wiley: Chichester.